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TITLE: The Role of SIRT1 in Breast Cancer Stem Cells

PRINCIPAL INVESTIGATOR: Songlin Zhang, MD, PhD

RECIPIENT: University of Texas Health Science Center at Houston,HOUSTON, TX 77030

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
SIRT1 inhibitors cambinol and Ex527 are used for treatment in several breast cancer cell lines, including T47D, TB549, MDA-MB-231 and MDA-MB-468. Stem cell markers, SOX-2 and Nanog, are significantly decreased in SIRT1 inhibitor treated cancer cells by qRT-PCR and western blot in T47D cell line. The ALDH positive cells in MDA-MB-231 cell line are significant reduced from 29% to 3.2% with Adeflour assay, and the CD44 expression is significantly reduced in CD44/CD24 flow cytometry analysis. Using qRT-PCR, SIRT1 inhibitors significantly down regulate vimentin (-3.7 folds), N-cadherin and smooth muscle actin (-2.7 folds) and up regulate the gene of E-cadherin, indicating SIRT1 inhibitors can block the epithelial mesenchymal transformation (EMT) of breast cancer. SIRT1 inhibitors can completely block TGF-beta induced EMT. SIRT1 inhibitors can significantly (60-70%) block the cancer cell invasion and migration in several triple negative breast cancer cell lines. SIRT1 inhibitors can significantly reduce the mammosphere formation in T47D cells. Immunohistochemistry performed on breast cancer specimens shows the correlation between cancer stem cell markers and SIRT1 overexpression. SIRT1 inhibitors can inhibit cell proliferation on all tested breast cancer cell lines, and can induce significant cell apoptosis in T47D cells. Cell cycle analysis shows tumor cells are blocked at G1 to S phase.

The molecular mechanism of SIRT1 inhibitors in blocking EMT and reducing cancer stem cells is likely associated with blocking the Wnt pathway. Several down stream target genes of Wnt pathway, such as cyclin D1, c-Myc and c-Jun, are significantly down regulated after using SIRT1 inhibitor cambinol, and the changes are more significant in TGF-beta stimulated cancer cells. Beta-catenin is significantly reduced including the active beta-catenin, and the decreasing beta-catenin protein may be related to the decreased Dvl proteins.

15. SUBJECT TERMS
SIRT1 inhibitors, cancer stem cell, epithelial mesenchymal transformation, invasion, migration, cell proliferation, apoptosis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Breast cancer stem cells (CSCs) are the subpopulation of cancer cells that are thought to be primarily responsible for drug resistance and cancer recurrence. In this study we will determine if SIRT1 inhibitors can be used as drugs to target breast CSCs. Specifically, we will see if SIRT1 inhibitors can block epithelial mesenchymal transformation (EMT) and can reduce breast CSCs and make them sensitive to traditional chemotherapy drugs. We will perform studies including in vitro study with breast cancer cell lines, human breast cancer tissue samples and in vivo study with xenograft mouse model.

2.

Cancer stem cell, epithelial mesenchymal transformation, SIRT1 inhibitor, xenograft model, signal pathway, immunohistochemistry, cell culture, breast cancer

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Task 1. SIRT1 inhibitors can induce differentiation of CSCs in breast cancer cell lines.
Task 2. Human breast cancer cells (from patient's samples) with CSC features have SIRT1.
Task 3. SIRT1 inhibition can decrease metastasis, induce differentiation of CSCs, reduce EMT, and increase tumor cell sensitivity to chemotherapy in xenograft mouse model.
Task 4. Wnt pathway is highly activated in breast CSCs and EMT of human breast cancer specimens.
Task 5. Wnt pathway is blocked in the SIRT1 inhibition xenograft tumor tissue, which is responsible for inducing differentiation of CSCs and reducing EMT.
Task 6. Using cell line *in vitro* study to demonstrate that SIRT1 regulates CSCs and EMT through activation of Wnt pathway via interaction with Dvl proteins.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

In the past year, we did studies to address tasks #1, #2 and #6, and just started in vivo animal xenograft study to address tasks #3 and #5. The IHC method for Wnt pathway (task #4) has been validated for beta-catenin, activate beta-catenin, phorphorylation GSK, c-Jun, cyclin D1 and c-Myc. The significant results include: 1) SIRT1 inhibitors block breast cancer EMT and reduce cancer stem cells; 2) SIRT1 inhibitors block Wnt pathway by affecting beta-catenin and DVL-3; 3) SIRT1 inhibitors reduce mammosphere formation in breast cancer cell lines; 4) SIRT1 inhibitors inhibit cancer cell proliferation and cause cancer cell apoptosis; 5) SIRT1 is over expressed in some breast cancer cells and appear related to cancer stem cells in cancer samples.

Task 1. SIRT1 inhibitors can induce differentiation of CSCs in breast cancer cell lines.

Breast cancer cell lines MDB-MA-231, MDB-MA-468, BT549, T47D and BT483 were purchased from ATCC, and SIRT1 inhibitors cambinol and Ex527 have been used for the in vitro study. SIRT1 inhibitors significantly reduced breast cancer stem cell population. Adeflour flow cytometry showed significantly decreased ALDH positive cells in cambinol treated MDB-MA-231 cells (3.2% vs 29.0% in controls), and CD44/CD24 flow cytometry study also showed significant down regulation cancer stem cell marker CD44 in cambinol treated MDB-MA-231 cells. The CD44/CD24 flow cytometry study on MDB-MA-468 showed completely loss CD24 activity after cambinol treatment. In T47D breast cancer cell line, cambinol treatment significantly down regulates stem cell regulative genes SOX-2 and Nanog by western blot and qRT-PCR analysis. Mammosphere in vitro functional study using T47D breast cancer cells also demonstrated significant decreasing mammosphere numbers and size after cambinol treatment.

In corresponding with the stem cell study, breast cancer cell invasion and migration studies also showed similar findings of SIRT1 inhibitors blocking cancer cell EMT. Cambinol showed 60-70% inhibition on breast cancer invasion in MDB-MA-231, MDB-MA-468 and BT549 cells, and it also significantly reduce cancer cell migration in wound healing assay.

In summary, in vitro study using several techniques with several breast cancer cell lines showed SIRT1 inhibitors block cancer cell EMT and reduce cancer stem cell population.

Task 2. Human breast cancer cells (from patient's samples) with CSC features have SIRT1.

Several breast cancer stem cell markers, including ALDH1a, CD44, CD133, SOX-2, Nanog and Oct-4, had been used in the pilot immunohistochemistry study on 20 breast cancer cases. ALDH1a/CD44 cocktail IHC has been used and a subpopulation cancer cells are positive for both markers. SXO-2 has been a good and useful marker in breast specimens. SIRT1 appears to be associated with the stem cell markers, but further study and more cases will be added.

Some tumor cells show very high SIRT1 expression in few cases after neoadjuvant or chemotherapy, and ALDH1a/CD44 IHC also show correlation with SIRT1.

Task 3. SIRT1 inhibition can decrease metastasis, induce differentiation of CSCs, reduce EMT, and increase tumor cell sensitivity to chemotherapy in xenograft mouse model.

The first xenograft mouse study has been started since April 5, 2014, and the study is ongoing and will be finished around June 12, 2014. SIRT1 inhibitor cambinol appear to reduce tumor growth in MDA-MB-231 xenograft mouse model by the early data.

Task 6. Using cell line *in vitro* study to demonstrate that SIRT1 regulates CSCs and EMT through activation of Wnt pathway via interaction with Dvl proteins.

SIRT1 inhibitor cambinol significantly down regulate Wnt down-stream target molecules, such as cyclin D1, c-Jun and c-Myc by both western blot and qRT-PCR in breast cancer cell lines. Western blot shows SIRT1 inhibitors block Wnt pathway by significantly reducing beta-catenin protein and DVL-3 protein.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

This project provides a postdoctal fellowship training position and the current fellow (Dr. Min Li) has opportunity to learn flow cytometry, cell culture, RT-PCR, western blot and some animal study skills. He is transforming from a clinical physician to a physician scientist.

This study also provides the PI (Dr. Songlin Zhang) opportunities to gain more laboratory management skills, to build collaboration with other basic scientists and clinical oncologists and breast oncology surgeon, to present the exciting data at several conferences and seminar, and to collect preliminary data for applying further grants (DOD, NIH and state grants).

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

A breast cancer research and clinical management team has been formed at UTHSC at Houston including breast surgeon, oncologists, radiologists and pathologists. The data have been presented regularly to the team member, and a grand round has been schedule for the oncology department. Several abstracts have been prepared to present in the national and international meeting next year.

The results from the first year will be reported in 2-3 peer reviewed scientific articles. The xenograft mouse study will be finished in 2-3 months next year, and morphoproteomic analysis with western blot and qRT-PCR will be performed in the tumor tissue (Task #3, #4 and #5). Continue evaluation of SIRT1 inhibitor in breast cancer treatment has been proposed for few grant applications including DOD breakthrough award. The goals are to have clinical trial as early as possible.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The early data from the first year study has confirmed that SIRT1 play significant role in breast cancer stem cells and cancer metastasis, and is the first study so far to show SIRT1 inhibitors can be used to reduce breast cancer stem cells. SIRT1 inhibitors can block TGF-beta induced EMT, and can inhibit tumor cell growth and lead to tumor cell apoptosis. Xenograft mouse study showed SIRT1 inhibitors significantly inhibited tumor growth on the early observation. The value of using SIRT1 inhibitor for breast cancer treatment has been confirmed by the cell line and xenograft study, and subclinical and clinical trial of using SIRT1 inhibitors for breast cancer treatment should be considered.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

It has been very difficult to evaluate the breast cancer stem cells in cancer sample and multiple markers appear useful in some studies but could not be confirmed by other studies. Combination with ALDH1a/CD44 double staining and SOX-2 stain provides a better tool for the evaluation of breast cancer stem cells. Large scale study is necessary for the validation.

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*

- *improving social, economic, civic, or environmental conditions.*

Several high school students and medical students have shown their interesting in breast cancer research and few of them did rotation or will do their rotation in my lab. Some visiting scientists from China also visited the lab.

5. CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to report.

ges in

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

SIRT1 as a therapeutic target in breast cancer. Department Research Seminar 03/21/2014

SIRT1 as a therapeutic target in breast cancer. Grand Round, medical oncology, UTHSC, 09/15/2014

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Dual color immunohistochemistry staining for ALDH1a/CD44, which has been used for other collaboration studies.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Songlin Zhang
Project Role:	PI
Nearest person month worked:	2
Contribution to project:	Design experiments, analyze data
Name:	Robert Brown
Project role:	Co-PI
Nearest person month worked:	<1
Contribution to project:	Analyze immunohistochemistry
Name:	Min Li
Project role:	Postdoc fellow
Nearest person month worked:	9
Contribution to project:	Perform experiments and analyze data
Name:	Baoxiang Guan
Project role:	Research associate
Nearest person month worked:	11
Funding support:	Department of Pathology, UTHSC
Contribution to project:	Perform experiments and analyze data
Name:	Pamela Johnston
Project role:	Research associate
Nearest person month worked:	1
Funding support:	Department of Pathology, UTHSC
Contribution to project:	Performing immunohistochemistry

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

Organization name: MD Anderson Cancer Center
Location of Organization: Houston, TX
Partner's contribution to the project: Facilities

8. SPECIAL REPORTING REQUIREMENTS

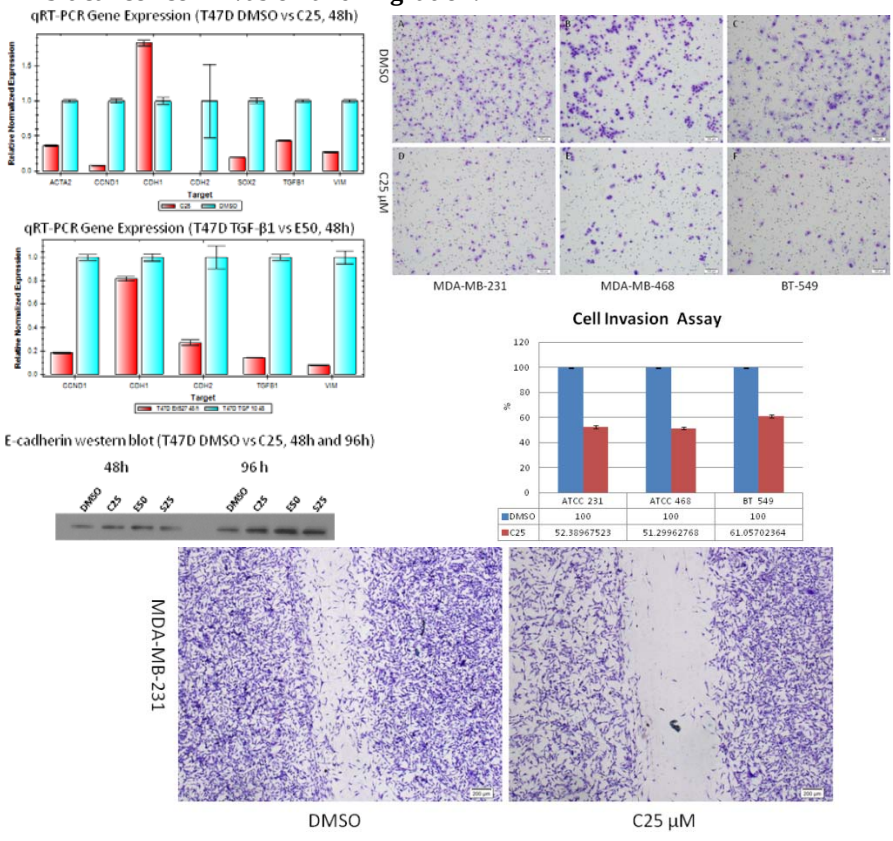
COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Preliminary Data

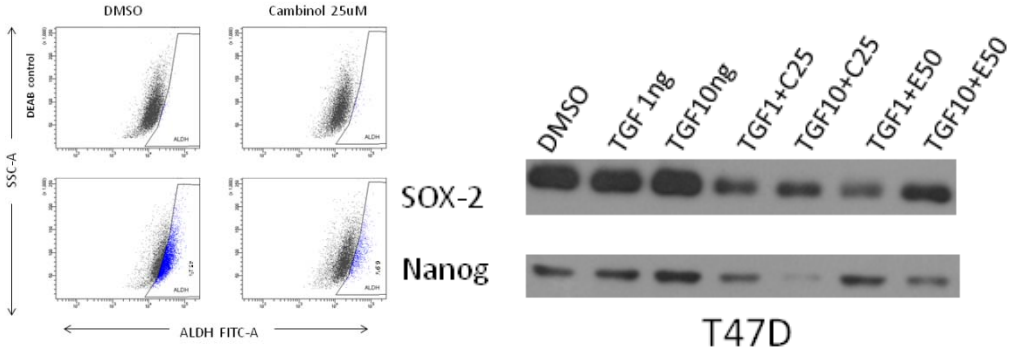
SIRT1 inhibitors cambinol and Ex527 can block epithelial mesenchymal transformation (EMT) in breast cancer cell lines and inhibit cancer cell invasion and migration.



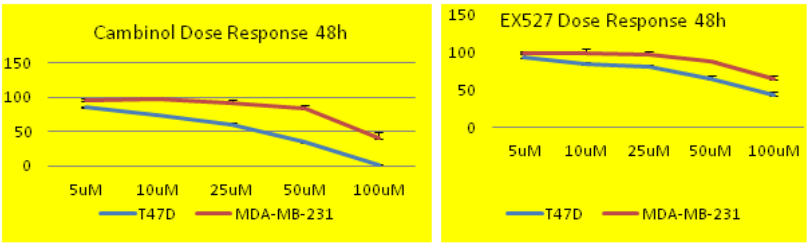
The left panel shows SIRT1 inhibitors cambinol and Ex527 significantly reduce the RNA levels of several EMT related proteins including vimentin, smooth muscle actin, and N-cadherin, and up-regulate epithelial marker E-cadherin in T47D cancer cells. The western blot shows increasing E-cadherin with the SIRT1 inhibitors. The right panel shows SIRT1 inhibitor cambinol can significantly block the cancer cell invasion in 3 triple negative breast cancer cell lines. The bottom figure shows cambinol also significantly reduce the migration of MDA-MB-231 cells.

SIRT1 inhibitors can reduce cancer stem cell population in breast cancer cell lines.

Cambinol reduces the ALDH positive cells from 45% to 6.9% in MDA-MB-231 cells (left), and the stem cell markers SOX-2 and Nanog are significantly down regulated after SIRT1 inhibitors (right).



SIRT1 inhibitors can inhibit cancer cell proliferation and cause tumor cell apoptosis.



Breast cancer cells show a dose response to SIRT1 inhibitors cambinol and Ex527, and cancer cells are more sensitive to cambinol than to Ex527. T47D cells have higher SIRT1 expression than MDA-MB-231, which is more sensitive to SIRT1 inhibitors.